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## 1 Symmetry breaking of tissue mechanics in wound induced hair follicle regeneration of

### 2 laboratory and spiny mice

- 3
- 4 Hans I-Chen Harn<sup>1,2</sup>, Sheng-Pei Wang<sup>1,2</sup>, Yung-Chih Lai<sup>3</sup>, Ben Van Handel<sup>4</sup>, Ya-Chen Liang<sup>1,3</sup>,
- 5 Stephanie Tsai<sup>1,5,6</sup>, Ina Maria Schiessl<sup>7</sup>, Arijita Sarkar<sup>4</sup>, Haibin Xi<sup>8,9</sup>, Michael Hughes<sup>2</sup>, Stefan
- 6 Kaemmer<sup>10</sup>, Ming-Jer Tang<sup>2,11</sup>, Janos Peti-Peterdi<sup>7</sup>, April D. Pyle<sup>8,9,12,13</sup>, Thomas E. Woolley<sup>14</sup>,
- 7 Denis Evseenko<sup>4,15</sup>, Ting-Xin Jiang<sup>1</sup>, Cheng-Ming Chuong<sup>1,\*</sup>
- 8
- 9 1 Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles,
   10 California, USA
- 2 International Research Center of Wound Repair and Regeneration (iWRR), National Cheng Kung
   University, Tainan, Taiwan
- 13 3 Integrative Stem Cell Center, China Medical University Hospital, China Medical University, Taichung,
   14 Taiwan
- 4 Department of Orthopaedic Surgery, Keck School of Medicine of USC, University of Southern
   California, Los Angeles, CA, USA
- 17 5 Ostrow School of Dentistry, University of Southern California, Los Angeles, California, USA
- 18 6 School of Dentistry, National Taiwan University, Taipei, Taiwan
- 19 7 Department of Physiology and Neuroscience, Zilkha Neurogenetic Institute, Keck School of Medicine,
- 20 University of Southern California, Los Angeles, CA, USA
- 8 Department of Microbiology, Immunology, and Molecular Genetics; University of California Los Angeles,
   Los Angeles, CA, USA
- 9 Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research; University of
   California Los Angeles, Los Angeles, CA, USA
- 25 10 Park Systems Inc. 3040 Olcott Street, Santa Clara, CA 95054
- 26 11 Department of Physiology, Medical College, National Cheng Kung University, Tainan, Taiwan
- 27 12 Molecular Biology Institute; University of California Los Angeles, Los Angeles, CA, USA
- 13 Jonsson Comprehensive Cancer Center, University of California Los Angeles, Los Angeles, CA, USA
- 29 14 Cardiff School of Mathematics, Cardiff University, Senghennydd Road, Cardiff, UK
- 30 15 Department of Stem Cell Research and Regenerative Medicine, University of Southern California, Los
- 31 Angeles, CA, USA
- 32
- 33 \* Corresponding author: <u>cmchuong@med.usc.edu</u>
- 34

#### 35 Abstract

36 Tissue regeneration is a process that recapitulates and restores organ structure and function. Although previous studies have demonstrated wound-induced hair neogenesis (WIHN) 37 38 in laboratory mice (Mus), the regeneration is limited to the center of the wound unlike those observed in African spiny (Acomys) mice. Tissue mechanics have been implicated as an 39 integral part of tissue morphogenesis. Here we use the WIHN model to investigate the 40 41 mechanical and molecular responses of laboratory and African spiny mice, and report these models demonstrate opposing trends in spatiotemporal morphogenetic field formation with 42 association to wound stiffness landscapes. Transcriptome analysis and K14-Cre-Twist1 43 44 transgenic mice show the Twist1 pathway acts as a mediator for both epidermal-dermal interactions and a competence factor for periodic patterning, differing from those used in 45 46 development. We propose a Turing model based on tissue stiffness which supports a two-scale 47 tissue mechanics process: (1) establishing a morphogenetic field within the wound bed (mm scale) and (2) symmetry breaking of the epidermis and forming periodically arranged hair 48 49 primordia within the morphogenetic field (µm scale). Thus, we delineate distinct chemomechanical events in building a Turing morphogenesis-competent field during WIHN of 50 laboratory and African spiny mice and identify its evo-devo advantages with perspectives for 51 52 regenerative medicine.

#### 54 Introduction

The ultimate goal of regenerative medicine is to restore the function and structure of the original tissue. Wound healing in adult humans and mice generally undergoes reepithelialization successfully yet fails to develop further, resulting in a scar with excess collagen and an absence of other skin appendages such as hair follicles. To facilitate regenerative wound healing, we look into skin development to recapitulate principles of hair follicular neogenesis.

Patterns form with the break of homogeneity and lead to the emergence of new structure 61 or arrangement<sup>1</sup>. In skin development, Turing reaction-diffusion was shown to be involved in the 62 periodic pattern formation of feathers and hairs<sup>2,3</sup>. Yet, before periodic patterning occurs, a 63 morphogenetic field competent for Turing mechanism must take place; this should have proper 64 cell density, the ability to secret morphogens, and appropriate morphogen receptors<sup>4</sup>. Within the 65 morphogenetic field, FGFs, Wnt/β-catenin and Edar<sup>5-7</sup> signaling activate the epithelial cells to 66 aggregate and form hair placodes, which later interact with the dermal condensate and 67 invaginate into the dermis to form the foundation of a hair follicle. This process is characterized 68 by a series of cohesive molecular signaling and also physical cellular events such as cell 69 70 aggregation, collective cell migration and proliferation. Wnt/β-catenin signaling has been shown 71 imperative to progress these cells into morphogenesis, in which a series of other signaling molecules such as Lef1, Sox2, Edar, Shh, MMP and Twist2 are also expressed<sup>8,9</sup>. 72

Dynamic mechanical changes also occur during morphogenesis<sup>10,11</sup>. For epithelial cells 73 to collectively migrate during morphogenesis, there must be an emergence of a local active 74 stress acting at cell-cell or cell-matrix interfaces that creates an anisotropic force field<sup>12-14</sup>. Force 75 generation by myosin-II motors on actin filaments have been shown to drives cell and tissue 76 morphogenesis in drosophila embryonic development<sup>15</sup>. In other words, in order for hair placode 77 to form and invaginate, the epithelial cells must overcome the physical barrier provided by the 78 79 dermal cells and the extracellular matrix (ECM) in order to invaginate into the dermis. While 80 previous studies uncovered mechanisms that can turn on/off hair follicle development, the chemo-mechanical dynamics that allows epithelial placode to form and invaginate into the 81 82 dermis is largely unknown. Previously, Oster, Murray and Harris constructed a mathematical model that described the action of motile cells that could produce stress on their environment 83 and thereby produce heterogeneous spatial patterns through mechanical means<sup>16</sup>. This basic 84 theory assumes there are two populations: a motile population of cells, n, which can produce 85 stress; and a tissue substratum, the ECM, which has density. Critically, the ECM is treated as a 86 viscoelastic material, meaning that the ECM will deform subject to the traction forces produced 87 88 by the cells. Critically, if the forces are added and removed quickly the ECM will relax back to its original shape. The convergence of these morphological and molecular asymmetries lead to the 89 formation of dermal condensations (DC)<sup>17</sup> and activation of  $\beta$ -catenin in the adjacent epidermal 90 cells to initiate feather bud gene expression<sup>18,19</sup>. 91

In the avian skin development, the early formation of the morphogenetic field is recognized as the feather tract field, and individual buds form sequentially or simultaneously within the tract field, with some species-based differences<sup>4,18,20</sup>. This implies there are different ways to make competent morphogenetic fields<sup>21</sup>, while the region outside of the tract field becomes the apteric region. Thus, in skin development, periodic generation of skin appendages occur in two steps: first the formation of morphogenetic field and then the periodic patterning of cell collectives within the field.

99 Wound-induced hair follicle neogenesis (WIHN) is a regenerative outcome of wound healing where fully function hair follicles develop de novo from the center of large full thickness 100 excisional wounds<sup>22</sup>. This observation was originally described in rats, rabbits, sheep and even 101 humans, and investigated in depth in recent years<sup>23-26</sup>. Cells in wound bed may use different 102 paths to reach the morphogenetic competent state. We contemplate that to regenerate new hair 103 follicle in the wound bed of the adult skin, a morphogenetic field also has to be established first, 104 which then allows periodically arranged hair germs to be generated. Since adult cells have 105 different epigenetic landscapes from embryonic cells, the generation of hair placodes in the 106 107 adult may not follow the exact pathway as in development. Investigating the chemo-mechanical dynamics of the epithelial cells and the ECM during hair follicular neogenesis would facilitate our 108 understanding on how to set up the morphogenetic field and initiate the signaling events, which 109 110 would have translational potential.

In search for the ultimate regeneration model, the African spiny mouse (Acomys 111 cahirinus) serves as an adequate model to study complete skin regeneration. The spiny mouse 112 has evolved to give away up to 70% of its skin to its predator and still remarkably regenerates 113 its entire skin and appendages<sup>27-29</sup>. Its skin is mechanically softer than the laboratory mouse (20 114 times differences) and much easier to break (77 times less the energy required than the 115 laboratory mouse)<sup>27</sup>. The gene expression profiles of the spiny mouse wound show a dampened 116 response of collagen. MMP and inflammatory molecules after wounding, suggesting an 117 alternative microenvironment to enrich hair neogenesis<sup>30</sup>. On the other hand, studies in 118 laboratory mice have shown that traction, or tension across the skin or wound, causes 119 hypertrophic scar through FAK signaling<sup>31</sup>; implying the significance of tensile state of the connective tissue to tissue functioning<sup>32,33</sup>. Nevertheless, the initiating events and how these 120 121 findings can translate into our understanding in laboratory mice (*Mus musculus*) and human 122 remain to be explored. Thus we hypothesize that spatial tissue mechanics of the wound partake 123 124 in establishing the morphogenetic field for hair follicular neogenesis, and there lies a chemo-125 mechanical signaling event that initiates the symmetry breaking of the epidermis and leading to placode formation and invagination. We show Twist 1 pathway plays a key role in modulating 126 127 tissue stiffness and facilitate hair formation. Furthermore, by delineating the common and distinct features of laboratory and spiny mouse during WIHN, we learn from evo-devo 128 advantages to provide perspective for future implications. 129

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## 134 Results

135 Tissue mechanics set up morphogenetic field for wound-induced hair neogenesis. To examine the effects of tissue mechanics on wound induced hair neogenesis, we first created 1 x 136 1 cm full thickness wounds on the dorsal skin of 3-week-old C57Bl/6 mice, and observed new 137 hair follicles formed at the center of the wound on post-wound day 28 (PWD28, Fig. 1a-c). To 138 investigate the spatial stiffness distribution of the wound bed, we used an atomic force 139 microscopy (AFM)<sup>34</sup> to measure across the wound (Supplementary Fig 1a), and calculated 140 tissue stiffness from force-displacement curves (Supplementary Fig 1b-c) using a modified 141 Hertz model<sup>35,36</sup>. We found that the center of the PWD14 wound, where *de novo* hair follicles 142 can be observed, is significantly softer than the wound periphery ( $28.0 \pm 1.1 \text{ vs} 10.5 \pm 0.6 \text{ kPa}$ , 143 144 Fig 1d-e).

To evaluate whether the observed tissue stiffness plays a role in hair follicle neogenesis, 145 we treated the wound daily from PWD10 to PWD16 with 100 µM of Blebbistatin (Fig. 1f), and 146 147 observed an increase in the number of hair neogenesis (Fig. 1g-h) and decrease in the stiffness 148 of the wound bed (Fig. 1i). Statistically, Blebbistatin significantly increased the number of resultant hair follicles from 17.4 ± 2.1 to 34.5 ± 4.3 kPa (Fig. 1j, n=8), and significantly increased 149 the areas of the wound bed that is under 15 kPa from 2.38  $\pm$  0.13 to 3.70  $\pm$  0.26 mm<sup>2</sup> 150 (Supplementary Fig 1d). To correlate tissue stiffness with hair neogenesis, we quantified and 151 plotted the area of the wound under 15 kPa versus the number of hair neogenesis observed in 152 each wound, and found a strong correlation ( $R^2 = 0.8082$ ), suggesting stiffness may contribute 153 to creating the morphogenetic field for hair neogenesis (Fig. 1k). 154

We further explored the molecular constituents of the wound stiffness. Collagen has 155 been implicated as the main extracellular matrix (ECM) of the wound<sup>37</sup>, hence we used second 156 harmonic generation (SHG) to visualize the amount and organization of collagen fibrils in the 157 wound (Fig. 1I-g). Generally speaking, fibers in PWD14 wounds are thinner and less dense than 158 159 in PWD21 ones. Both the fiber density and thickness are significantly higher in the wound margin than in the wound center in PWD14 and PWD21 (Fig. 1r-s). Hence, the spatiotemporal 160 organization of collagen fibrils in the wound bed on PWD14 and PWD21 corresponds to its 161 respective stiffness of the wound (Fig. 1t). This implies the wound bed needs to be softer than 162 15 kPa for hair neogenesis to occur (green bar, Fig. 1t). Blebbistatin treatment not only softened 163 the entire wound bed, but also more importantly, significantly lowered the stiffness of 164 165 intermediate margin-center region of the wound (blue and purple, Fig. 1t), hence setting up a larger morphogenetic field competent for hair neogenesis (green, Fig. 1u). These results 166 suggest tissue mechanics play an important role in hair neogenesis, and the spatial organization 167 of collagen fibrils may be the main constituent of the tissue stiffness in the wound bed. 168

169 African spiny mouse exhibits an optimal range of tissue stiffness for placode formation.

The African spiny mouse is known to have robust ability in WIHN (Fig. 2a). Unlike laboratory

mice that show *de novo* hairs only in the center of the wound bed, new hair follicles were

observed across the entire wound bed on PWD28 (Fig. 2b-c). To explore how these events
 occur over time, we examined the spatiotemporal pattern of hair placode emergence in the

spiny mouse. Interestingly, we found an opposite trend. In spiny mice, the hair placodes started

to develop from the periphery of the wound bed on PWD14 (Fig. 2e, e1-3). The center of the

wound bed did not form hair placodes until PWD21 (Fig. 2f). How can we explain this oppositetrend? Can tissue mechanics play a role?

We used an AFM to determine the spatiotemporal dynamics of tissue stiffness during 178 179 WIHN in spiny mice (Fig. 2d). In general, two trends are similar to that of the laboratory mice: 1) the center of the wound bed is softer than the wound periphery (Fig. 2d), and 2) the overall 180 stiffness of the wound increased from PWD14 to PWD21 (Fig. 2e-f). However, there are two 181 182 major differences: 1) the soft nature of the unwounded spiny mice skin and wound bed, and 2) the periphery-to-center formation pattern of *de novo* hair primordia in the spiny mice wounds. 183 The unwounded spiny mouse skin and the stiffest region of the wound bed, the wound margin, 184 are still softer than 15 kPa, while the center of the wound bed fell below 5 kPa on PWD14 (Fig. 185 2e). As a result, the hair primordia formation in the spiny mouse wound followed a periphery to 186 187 central trend; the hair placodes only began to form where the wound stiffness was higher than 5 kPa (Fig. 2f). Hence, the *de novo* hair follicles were more mature at the wound periphery than at 188 the wound center, and this morphological feature is apparent on PWD28 (Fig. 2g). This result is 189 190 best explained by the presence of an optimal range between 5-15 kPa of tissue stiffness for placode formation. To test this hypothesis, we further softened the spiny mouse wound bed by 191 Blebbistatin treatment and showed that: 1) Blebbistatin treatment softened the wound bed and 192 193 significantly increased the wound area under 5 kPa (Supplementary Fig 2a-c), and 2) the resultant number of hair neogenesis was significantly reduced from  $74.00 \pm 6.14$  to  $12.40 \pm 1.10$ 194 (Fig. 2j, Supplementary Fig 2d). These results suggest that regions of wounds that are too stiff 195 or too soft are not favorable for new hair formation. 196

What is the molecular basis of these differences in tissue stiffness? To investigate the 197 structure and organization of collagen fibrils in the spiny mouse wound as observed in the 198 laboratory mice, we also used SHG to visualize it. Interestingly, collagen fibrils were identified in 199 the unwounded spiny mouse skin but almost undetectable within the wound center in both 200 201 PWD14 and PWD21 wounds (Fig. 2h). We analyzed and compared the fiber thickness and fiber 202 density of PWD14 and PWD21 spiny mouse wound margin (Supplementary Fig 2e-f), and 203 showed that the collagen fibrils in the spiny mouse wounds are significantly thinner and less dense than that of the laboratory mice (Supplementary Fig 2g-h). The spiny mouse skin has 204 been reported to have high levels of collagen III<sup>27</sup>; however, since type III are less crystallined 205 and generate little SHG signal<sup>38,39</sup>, we further used IHC to examine the expression of collagen I 206 and III in the spiny mouse wounds. We found that collagen III is the dominant collagen type 207 expressed in the early stages of spiny mouse wound healing (Fig. 2i); nevertheless, this 208 209 relationship is reversed later in post 16-week wounds when collagen I was highly expressed in contrast to collagen III (Supplementary Fig 2i). To summarize, we found that there is a lower 210 211 limit on the softness of the wound bed at 5 kPa in order for hair neogenesis to occur, as the 212 initial hair placodes can only be observed in wound stiffness between 5-15 kPa in both laboratory and spiny mice wounds (Fig. 2k). This implies that 5-15 kPa could be the optimal 213 214 range for the wound to set up the morphogenetic field for hair placode formation, and suggests why hair neogenesis begins from the wound periphery and to center later in the spiny mice (Fig. 215 216 21).

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### 218 **RNA-seq analysis identifies epidermal Twist1 as an upstream regulator for the formation**

- of hair placodes in WIHN. To delineate the molecular mechanism underlining WIHN,
- specifically at the morphogenetic field, we used a 5 mm punch biopsy to separate the PWD14
- 221 epidermis into the regenerative wound center and the non-regenerative wound periphery, and

performed RNA-seg analysis to look for differentially expressed genes (DEG) between the two. 222 PWD14 was selected because our preliminary data showed that gene expression relate to hair 223 neogenesis (Wnt5a, Lef1, Gli1, Fgf10 and Twist1) peaked in the wound center of PWD14 224 epidermis (Supplementary Fig 3a-b). From the RNA-seg analysis, we identify 2,780 DEG (Fig. 225 3a). Among them, Wnt/ $\beta$ -catenin signaling (P = 1.49 X 10<sup>-2</sup>), Integrin signaling (1.84 X 10<sup>-8</sup>), 226 Stat3 pathway (1.01 X 10<sup>-6</sup>), Inhibition of Matrix Metalloproteases (4.53 X 10<sup>-7</sup>), EMT core genes 227  $(4.23 \times 10^{-10})$ , proliferation of epithelial cells  $(3.47 \times 10^{-17})$ , cell movement of epithelial cells 228 (5.43 X 10<sup>-13</sup>), and organization of ECM are significantly enriched (Fig. 3b-c, Table 1). 229 Furthermore, we identify 20 significantly upregulated genes related to hair placode formation 230

231 (Table 2).

To identify the potential upstream initiators of WIHN, we identified 114 transcription 232 233 factors (TF) that are significantly upregulated at the wound center, including those that are related to the Twist (*Twist1*, *Twist2*, *Snai1*) and Wnt (*Tcf23*) pathways (Table 3). Among them, 234 Twist1 is the second most statistically significant upstream regulator, which regulates many 235 downstream DEG (P = 4.55 X 10<sup>-11</sup>) as indicated by the Upstream Analysis function in Ingenuity 236 Pathway Analysis (IPA) (Fig. 3d-e). The top significant transcription factor is *Nfkbia* (3.28 X 10<sup>-13</sup>) 237 which is associated with the inflammatory response after wounding (Fig. 3d). Wholemount 238 239 immunostaining also showed that epidermal Twist1 is highly expressed in the PWD14 wound center and enriched in both hair placode and inter-placode region more than in the margin (Fig. 240 3f, Supplementary Fig 3c). IHC staining of Twist1 and Snai1 (number 5 ranked TF) on PWD14 241 sections show both TF are expressed in the epithelial hair placode (Fig. 3g). These findings 242 243 point to ECM remodeling, cell proliferation, cell movement, Stat3, and Wnt/ $\beta$ -catenin as 244 important signaling events during WIHN, and epidermal Twist1 appears to be an important upstream regulator for hair placode formation. 245

Given the differences between laboratory and spiny mice during WIHN<sup>29</sup> (Fig. 1, 2) we 246 also performed RNA-seg analysis on spiny mouse wounds to identify key molecules for its hair 247 primordia formation. Since the entire spiny mouse wound bed is capable of undergoing WIHN, 248 we harvested the entire wound on PWD0, 14, 21, and 28 and separated them into epidermis 249 and dermis to analyze the quantitative changes of gene expression before, during early, mid, 250 and late stages of WIHN (Fig. 4a). From the wound epidermis, the proliferation of epithelial cells 251 and cell movement associated genes all peaked on PWD14, including Twist1 (Fig. 4b). In the 252 253 dermis, Twist1 and its related TF Zeb2 also peaked on PWD14, while Zeb1 and Tgfb1 showed a different trend (Fig. 4c). Interestingly, many of the ECM and MMP related genes along with 254 other TFs such as Stat3 are highly expressed on PWD21 and PWD28 (Fig. 4d), which may 255 256 reflect the softness of PWD14 spiny mouse wounds (Fig. 2k). IHC staining of PWD14 spiny mouse wounds also show that Twist1, Zeb2, MMP9 and P-cad are expressed around the hair 257 placode, while E-cadherin is downregulated at the hair placode. As suggested by its RNA-seq 258 analysis, Twist1, Zeb2 and MMP9 are also expressed in the dermis (Fig. 4e). Lastly, using 259 260 Harmine and GM6001, we show that inhibiting Twist1 and pan-MMP activity in the spiny mouse 261 wounds also significantly reduced WIHN (Fig. 4f-g).

It should be noted that while dermal Twist1 is highly expressed in embryonic skin, it is absent in the E14 epidermal hair placode (Supplementary Fig 3d). Identifying epidermal Twist1 in WIHN suggests that the spatiotemporal dynamics of the tissue stiffness and gene expression may play a more important role in the epithelial placode formation in WIHN than in embryonic development.

Epidermal Twist1 regulates both epidermal and dermal cell behavior and tissue stiffness 267 toward hair primordia formation in WIHN. To verify the role of epidermal Twist1 in WIHN, we 268 crossed the K14-Cre mice with Twist1-loxP mice to generate the K14-Cre-Twist1 mutant mice 269 270 (Fig. 5a) that expressed little Twist1 in the epidermis (Supplementary Fig 4a). The mutant mice showed a significant decrease in hair neogenesis (Fig. 5b-c, n), and a significantly stiffer wound 271 272 bed than that of wild type mice (Fig. 5d, o, Supplementary Fig 4b). We further used small molecule inhibitors to suppress Twist1 (Harmine, Supplementary Fig 4a) and its downstream 273 MMP activities (GM6001), and both treatments significantly decreased the number of hair 274 275 neogenesis (Fig. 5e-j, n), stiffened the wound center (Fig. 5o, Supplementary Fig 4b), and 276 reduced the wound area under 15 kPa (Supplementary Fig 4c). Interestingly, in the perturbed wounds, the wound area under 15 kPa showed a correlation with the resultant number of hair 277 follicles ( $R^2 = 0.7496$ , Supplementary Fig 4d), although the slope of the trend line is much 278 smaller than that of the control and Blebbistatin treated samples (1.6828 vs 12.812, 279 Supplementary Fig 4d and Fig. 1k). 280

281 We further used lentivirus to transfect and overexpress Twist1 in the wild-type mouse wounds on PWD10 and observed a significant increase in the resultant hair follicle numbers on 282 PWD28 (Fig. 5k-n). Correspondingly, we re-analyzed public microarray data that compared 283 mouse strains with high and low WIHN capacity<sup>40</sup>, and found that Twist1 expression levels are 284 also significantly higher in the high regenerative strain (C57BL/6 X FVB X SJL) than the low 285 regenerative one (C57BL/6, p = 0.004, Supplementary Fig 4e). These findings verify that Twist1 286 and its downstream signals such as MMP play important roles in controlling wound stiffness and 287 288 hair follicle neogenesis during WIHN (Fig. 5n-o).

289 To explore the signaling molecules perturbed by Twist1 knockdown during WIHN, we compared the gene expressions of PWD14 wild type epidermis wound center to that of K14-290 Cre-Twist1, and found downregulation in many genes related to cell proliferation, Wnt/β-catenin 291 signaling (Supplementary Fig 4f-g) and ECM remodeling (Fig. 5p). Previous studies have 292 demonstrated that the epidermal placodes are required for underlying dermal condensation and 293 the ensuing hair follicle development<sup>41,42</sup>. To further explore whether epidermal Twist1 affects 294 DC fate acquisition during WIHN, we performed RNA-seq analysis on PWD14 wild type dermis 295 wound center, PWD14 wild type dermis wound margin, and PWD14 K14-Cre-Twist1 dermis 296 wound center. There are 1623 DEG between PWD14 wild type dermis wound center vs margin 297 (Supplementary Fig 5a). Among them, DC signature genes are significantly enriched (p = 1.06 298  $X 10^{-5}$ ), which suggests DC niche formation in the PWD14 dermis wound center. In addition, we 299 found 928 DEG between K14-Cre-Twist1 and wild type dermis wound center comparison 300 301 (Supplementary Fig 5b), and the significantly enriched pathways include cell movement, ECM, inhibition of MMP, Wnt/β-catenin signaling and integrin signaling (Fig. 5g). To further investigate 302 the contribution of epidermal Twist1 to dermal condensation, we overlapped DEG from wild type 303 dermis center vs margin (total 1623 genes) and those from K14-Cre-Twist1 vs wild type dermis 304 center genes (total 928 genes) (Supplementary Fig 5c), and found 258 significantly overlapping 305 306 DEG ( $p = 1.37 \times 10^{-40}$ ). This indicates many DEG between wild type dermis wound center and margin were perturbed by epidermal Twist1 knockout. Among these DEG, most of the 307 significantly upregulated genes were significantly downregulated by knocking out epidermal 308 Twist1, and vice versa (r = -0.61, p =  $1.1 \times 10^{-27}$ , Supplementary Fig 5d). Similarly, we can 309 identify 23 DC signature genes that are upregulated in the wild type dermis wound center vs 310 wound margin, which were also downregulated by epidermal Twist1 knockout (Supplementary 311 312 Fig 5e). In total, there are 31 DC signature genes that are downregulated in PWD14 K14-CreTwist1 dermis center versus wild type (Fig. 5r). These results suggest that epidermal Twist1 plays an essential role in regulating DC and following hair follicle regeneration via an epidermal-

dermal signaling interaction during WIHN.

Our findings here suggest tissue mechanics and epidermal Twist1 may feed in Wnt/β catenin based hair primordia formation pathway in WIHN. This new "non-canonical" concept will
 be further discussed in discussion, together with literature.

319 Turing-like mechanism explains an optimal wound stiffness range facilitates new hair placode formation. From our previous mechanical analysis, we allude that an optimal range of 320 wound stiffness is required for hair neogenesis to occur. This also suggests that in order for the 321 322 hair placode to form and invaginate into the dermis, the aggregated cells should be able to overcome the physical barrier of the dermis. To quantify its mechanical properties, we used the 323 AFM to map multiple 100 x 100 µm squares in the wound center to explore the apparent 324 stiffness of the PWD14 hair placodes versus surrounding wound bed on a micrometer scale (Fig. 325 6a, left). The results show that the stiffness of hair placodes (17.36 ± 0.34 kPa) is significantly 326 327 higher than that of the wound center  $(10.53 \pm 0.58 \text{ kPa})$ , but still much lower than the wound margin (Fig. 6a, dot plot), implying that the activated epithelial placode cells also undergo 328 329 mechanical changes in addition to gene expressions (Fig. 6a'). In parallel, we also found that 330 the average stiffness of E14 mouse embryonic skin to be  $7.3 \pm 0.6$  kPa while its hair placode is 331  $9.6 \pm 0.5$  kPa (Supplementary Fig 6a-b), which is comparable to the microenvironment of the wound center in laboratory mice and the entire wound bed in spiny mice. 332

Turing model has been proposed as the underlying mechanism of pattern formation<sup>43</sup>. Here we look to construct a Turing system<sup>44</sup> to explain the differential placode formation pattern in laboratory and spiny mice, which is linked to the underlying structure of the solution region. Namely, the system should produce spots (hair placodes) within a specific region of stiffness; if the solution region is too soft, or too stiff, then the system does not pattern.

We consider three diffusible populations an activator, u, an inhibitor, v, and a measure of stiffness, E. Specifically, u, v and E are thought to be biochemical populations that are able to interact with each other. The prototypical "Schnakenberg" Turing kinetics<sup>43,45,46</sup> exist between the populations u and v. The Schnakenberg kinetics are a general form of Turing kinetics, whereby all dynamics can be connected to source parameters,  $\alpha$  and  $\beta$ . Since we have no guidance on kinetics these are as good as any kinetic type and swapping them for some more accurate kinetics should not influence the resulting conclusions.

We adapt the Schnakenberg kinetics by modulating the inhibitor source by the population *E*, which we take to be a stiffness measure of the field. Namely, a soft field has low density ECM and, thus, (we assume) the soft field produces more *E*, which, in turn produces more *v*. We consider a square spatial domain  $[-50,50] \times [-50,50]$ , centered at zero, with Neumann boundary conditions and random initial conditions. In terms of the mathematics we produce the following system of interactions:

$$\frac{du}{dt} = \underbrace{\nabla^2 u}_{\text{Diffusion of } u} + \underbrace{\alpha - u + u^2 v}_{\text{Schnakenberg interaction}}, \quad (1)$$
Rate of change of  $u$ 

$$\frac{dv}{dt} = D_v \underbrace{\nabla^2 v}_{\text{Diffusion of } v} + \underbrace{E - u^2 v}_{\text{Schnakenberg interaction}}, \quad (2)$$
Rate of change of  $v$ 

$$\frac{dE}{dt} = D_E \underbrace{\nabla^2 E}_{\text{Diffusion of } E} + \underbrace{SS(x, y)}_{\text{Region of}} - \underbrace{E}_{\text{decays over time}}. \quad (3)$$
Rate of change of  $E$ 

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The coefficients  $D_v$  and  $D_E$  are positive constants, which measure how quickly the populations spread. The source coefficient, *s*, measures the strength of the *E* source,

$$S(x,y) = \exp\left(-\left(\frac{x}{\sigma}\right)^2 - \left(\frac{y}{\sigma}\right)^2\right).$$
(4)

354

This means that the *E* source is a scaled Gaussian distribution centered at zero. The source is highest in the center and decays towards the boundary. Physically, this means that the tissue is softest in the center and stiffest on the boundary. The 'size' of the soft region is controlled by  $\sigma$ , namely, increasing  $\sigma$  makes the source function 'wider' meaning that more of the tissue is soft. An illustrative example of *S* is shown in Fig. 6B.

360 Simulations of increasing s can be seen in Fig. 6B. The top row (pick background) 361 shows the output *u* after a threshold (color change) has been applied, illustrates the change in tissue stiffness. The bottom row (blue background) shows the accompanying profile of E, 362 reflects spot pattern formation. Namely, the spots in the blue background illustrate the regions in 363 which u > 3.8. Once again, as E decreases (from pink to green and blue) we expect the tissue 364 beneath to be softer. Thus, we see that as *s* increases left to right the center becomes lighter 365 366 and lighter (pink background blocks). Notably, as the center becomes too soft (the last 2 pink background blocks in the third row) we see that placodes stop forming in this region (the last 2 367 blue background blocks in the bottom row). Hence, we have produced a simulation in which a 368 Turing pattern has a feedback loop with an external field, which is considered to be a measure 369 of the underlying ECM stiffness. Critically, in order for spots to form the media can be neither 370 too stiff nor too soft. 371

372

#### 373 Discussion

In summary, we show that multiscale tissue mechanics of the wound bed partakes in setting up the morphogenetic field for WIHN, and Twist1 is an important chemo-mechanical regulator involved in initiating cellular events that lead to placode formation and invagination through symmetry breaking of the epidermis, ECM remodeling, collective migration and epidermal-dermal crosstalk. As we try to recapitulate the developmental process to facilitate regenerative wound healing, the mechanical microenvironment of the tissue should also be considered. The stiffening of the epithelial hair placode serves as an important symmetry
 breaking point - cells invaginating into the soft dermis (Fig. 6a). Identifying 5-15 kPa as the
 optimal stiffness range (Fig. 2k) is important in setting up the morphogenetic field for the hair
 placodes to overcome the physical barrier of the microenvironment and invaginate into the
 dermis. Furthermore, different spatiotemporal dynamics of wound stiffness in different species
 predicate distribution of morphogenetic field and placode formation (Fig. 6c-d).

386 We demonstrate that there are two levels of symmetry breaking during successful wound induced hair neogenesis (WIHN), in parallel to the developmental process. The first level 387 is the generation of morphogenesis competent field (green) from the center (Fig. 6b-c). The 388 second level is the generation of periodically arranged hair germs forming (brown dots, Fig. 6b, 389 blue blocks) from the morphogenesis competent field. In the spiny mice, the topology is 390 391 reversed with the competence zone (green) on the periphery, while the central field (pink) cannot form hairs (Fig. 6c). By perturbing tissue stiffness, we can even generate a concentric 392 ring-shaped competent field, fulfilling the prediction of the model (Fig. 6b, d). Additionally, the 393 394 Turing mechanism can also help explain the asymmetric field in the less uniform environment (e.g. wound), development and growth<sup>47</sup>. 395

The key question is what factors are required to make a region competent to undergo further periodic Turing patterning to generate hair placodes in the adult skin. The multiscale tissue mechanics perspective allows us to compare the similar and distinct pathways in development and WIHN, and appreciate that laboratory and spiny mice have evolved and manifested during regenerative wound healing, in contrast to repair. The findings and concept, together with those in recent WIHN studies are discussed in the following.

Macroscale symmetry breaking of tissue mechanics in the wound bed leads to the emergence of morphogenetic field. By comparing the mechanical and molecular response of the laboratory and spiny mice during wound healing, we found that: 1) the hair placodes begin to form from the wound margin as early as PWD14 in spiny mouse, 2) a wound bed with stiffness between 5-15 kPa is optimal for hair neogenesis, and 3) the Twist1-related TFs are expressed on PWD14 in both laboratory and spiny mice, but additionally in spiny mice some other ECM, MMP, integrins and TF genes are upregulated on PWD21.

409 We postulate that the spatiotemporal formation of the hair placodes is a good indication of the morphogenetic field of the wound bed, established partly by tissue mechanics. Previous 410 studies have shown that there is an optimal matrix stiffness for different cell behaviors (e.g. cell 411 migration, proliferation, differentiation), and very soft matrix impedes focal adhesion formation 412 and cell migration<sup>48</sup>. We postulate that on PWD14 the wound center of the spiny mouse was still 413 too soft for epidermal cells to form the hair placode, a process that requires epithelial cells to 414 migrate and invaginate into the dermis, hence it occurred later on PWD21 when the wound 415 stiffness reached over 5 kPa. On the other hand, a wound bed stiffer than 15 kPa may be too 416 417 stiff for the epidermal hair placode cells to invaginate, as demonstrated by the thick and dense collagen fibers in the PWD14 laboratory mouse wound margin (Fig. 1I-s). The high collagen III 418 419 expression in the spiny mouse wound in PWD14 and PWD21 (Fig. 2i), in contrast to the very few detectable collagen fibrils by SHG (Fig. 2h, Supplementary Fig 2e-f), suggest that collagen 420 III (not very crystallined and generates little SHG signal<sup>38,39</sup>) may be responsible for constituting 421 the soft wound bed that resembles the physical environment of the embryonic skin, ideal for hair 422 423 neogenesis. Recent studies also showed that ECM stiffness and mechanical forces exerted from the epidermal cells cohesively instruct tissue architectures and function<sup>49,50</sup>. 424

Having a soft wound bed may also modulate the signaling of ECM remodeling gene 425 expression. The time-course RNA-seg analysis of the spiny mouse wound showed an 1-week 426 delay in the peak expression time of Twist1-related genes (PWD14) and ECM, MMP, integrins 427 and other TFs (PWD21) in spiny mouse (Fig. 4b-d). We speculate that a suitable tissue stiffness 428 is also required for TF to enter the nucleus, as supported by findings that force-induced nuclear 429 deformation modulates nuclear entry of TF<sup>51</sup>. Hinz has also proposed a candy wrap theory to 430 describe that certain level of mechanical force is required to release active TGF-ß from its latent 431 form<sup>52</sup>. The spinv mouse dermal cells also demonstrate fewer α-SMA-positive stress fibers upon 432 substrate stiffness increase<sup>53</sup>. The spiny mouse's ability to keep the wound bed soft during early 433 stages of wound healing could delay the nuclear entry and activation of the upstream TF and 434 hence the expression of certain ECM, MMP and integrins, and consequentially set up the 435 436 optimal molecular and mechanical wound bed as morphogenetic field. Furthermore, it is worth noting that Twist1 expression is higher in the dermis than epidermis (Fig. 4e) in spiny mouse 437 around the hair placode, which is more representative of the embryonic dermal Twist1 438 expression during development (Supplementary Fig 3d). These features could be the 439 440 evolutionary advantages that the spiny mice have evolved to promote regenerative wound 441 healing and survival. These similar yet distinct regulations of Twist1 and tissue mechanics between laboratory and the spiny mice remain to be investigated. 442

Microscale symmetry breaking of tissue mechanics in the morphogenetic field 443 leads to the emergence of hair primordia. We have identified Twist1 as the key upstream 444 chemo-mechanical regulator to activate ECM remodeling, epithelial cell movement, epithelial 445 446 proliferation, Wnt/ $\beta$ -catenin signaling, and DC in the dermis (Fig. 3-5). Analysis from a previous 447 microarray database also showed that Twist1 expression is higher in the mice strain with high regenerative capacity compared to a low capacity strain (Supplementary Fig 4e)<sup>40</sup>. Twist1 is 448 shown to directly regulate Cdh11, Grem1, Zeb1, Dkk3, Gli1, Fgfr1, Tbox18, Col6a2, and Lamb1 449 450 (Supplementary Fig 7) when we reanalyzed a database that used H3K4me3 ChIP-seg to show the epigenetic reprogramming following Twist1-mediated EMT in human epithelial cells<sup>54</sup>. 451 Others have also shown direct transcriptional binding of Twist1 on the Snai2 promoter<sup>55</sup>. This 452 effect of Twist1 also corroborates with the detected increase in the stiffness of the placode. At 453 the same time, Twist1 has also been shown to directly bind to the MMP promoter to exert its 454 transcriptional effect<sup>56</sup>. By inducing MMP activity at the hair placodes and remodeling local ECM, 455 we postulate this can lower the physical barrier of the dermis to also facilitate placode 456 invagination. In this study, we used small molecule inhibitors Harmine and GM6001 to inhibit 457 Twist1 and pan-MMP activities, respectively. Harmine targets the Twist1 pathways through its 458 promotion of Twist1 protein degradation<sup>57</sup> and is also capable of blocking the activities of dual-459 specificity tyrosine phosphorylation-regulated kinase (DYRK) family proteins and mitogen 460 activated protein kinase<sup>58</sup>. GM6001 is a potent reversible broad spectrum inhibitor of zinc-461 containing proteases, including various MMPs (MMP-1, -2, -7, -8, -9, -12, -13, -14, -16, and -26), 462 disintegrin and metalloproteinase domain-containing (ADAM) proteins ADAM9, ADAM10, 463 ADAM12, and ADAM17<sup>59</sup>. Although the results of the inhibitor treatment fell in line with our 464 hypothesis and other Twist1-functional perturbation studies (lentivirus transfection and K14-Cre-465 Twist1 transgenic mice), and there is no significant difference between the number of new hair 466 follicles observed in K14-Cre-Twist1 and Harmine treated wounds (Fig. 5n), we acknowledge 467 these inhibitors' potential side effects outside of Twist1 pathway activities. 468

Furthermore, in order for the epithelial cells to continue to invaginate downward into the dermis, the cell number needs to increase, which can be observed in the highly enriched 471 epidermal proliferation and movement genes in the gene set enrichment analysis (Fig. 3b). Similar expression of Twist1-related genes at the hair placodes have also been observed in our 472 spiny mouse RNA-seg analysis (Fig. 4b), others' laboratory mice WIHN microarray database<sup>60</sup> 473 and human hair follicle morphogenesis<sup>40</sup>. Our K14-Cre-Twist1 RNA-seq analyses also imply that 474 epidermal Twist1 plays a role in dermal-epidermal interaction (Fig. 5g-r). The well-established 475 morphogenesis initiator of skin development, β-catenin, has been shown to directly activate 476 Twist1 expression in skull progenitor cells<sup>61</sup>. Therefore, we reason that Twist1 is one of the 477 chemo-mechanical regulators that responds to  $\beta$ -catenin activation to induce symmetry breaking 478 of the morphogenetic field of the epidermis, which facilitates dermal-epidermal interactions and 479 480 initiates ECM remodeling, cell proliferation and collective migration, leading to placode formation and invagination during WIHN. Alternatively, Twist1 itself could also play the role of a 481 mechanosensor during mechanotransduction for the wound induced hair follicle neogenesis<sup>62-65</sup>. 482

WIHN studies identify the concept of non-canonical and canonical hair primordia 483 formation pathway. WIHN is a combination of local periodic patterning events and a global 484 influence that constitute the morphogenetic field. The canonical Wnt, β-catenin and Shh<sup>66</sup> have 485 been identified as the critical activators of WIHN. Ablation of Wnt in the wound epidermis via 486 inducible  $\beta$ -catenin deletion eliminates hair neogenesis, while overexpression of Wnt in the 487 wound epidermis enhances it<sup>22,67</sup>. On the other hand, non-canonical signaling also regulates 488 WIHN, some by interacting with the canonical signals. The  $\delta \alpha T$  immune cells secret Faf9 to act 489 490 on the neighboring myofibroblasts in the wound, inducing them to secret Wnt2a ligand<sup>60</sup>. Double stranded RNA (dsRNA), which is released during injury, activates Toll-like receptor 3 (TLR3)<sup>40</sup> 491 and its downstream effectors IL-6<sup>68</sup> and Stat3 to promote hair neogenesis. This effect is 492 493 achieved through the induction of known hair morphogenetic molecules such as Edar, Wnt and Shh pathways. 494

Based on our earlier developmental studies of periodic formation of the feather and hair 495 germs, we noticed there are two waves of molecular expression, which we name them 496 restrictive and *de novo* mode, respectively<sup>4</sup>. In short, restrictive mode molecules are present 497 before periodic patterning occurs, and are required for periodic patterning process, while de 498 499 novo molecules are the readout. Our data showed Twist1 is expressed higher in the wound center (Fig. 3f), it is not exclusively expressed in the nucleus of hair placode cells, but also inter-500 follicular epidermis (Fig. 3f-g). These results suggest that Twist1 belongs to the "restrictive 501 502 mode" molecules, and therefore they are present in the epidermis in both putative placode and 503 inter-placode regions. Our view is that Twist1 is initially broadly expressed in the morphogenetic 504 zone of the wound bed, and become accentuated in the placode region and enter the nucleus 505 when the sum of all the upregulating factors for placode formation reaches a threshold. This is supported by our experiments where either softening the wound bed or overexpressing Twist1 506 507 in the wound enhanced WIHN. In spiny mice, we note that epidermal Twist1 is expressed 508 predominantly but not exclusively in basal cells (Fig. 3f-g). While we postulate that activation of 509 Twist1 in the basal cells suggests a prelude for EMT, recent discovery in the heterogeneity of wound epidermal cells<sup>69</sup> eludes that a more comprehensive and single-cell resolution future 510 study is required to identify the molecular identities of these Twist1-positive and placode forming 511 512 cells.

In this study, we show that Twist1 plays a key role in epidermal and dermal signaling during wound-induced hair primordia formation, which is a different mechanism adopted during developmental process. Studies have identified the binding sites for β-catenin on Twist1 promoter<sup>61</sup>, although in our K14-Cre-Twist1 gene expression analysis, knocking out Twist1 also affected Wnt/β-catenin expression in both the epidermis and dermis during WIHN. We postulate

- 518 β-catenin may be the initial activator of Twist1, and Twist1 can also loop back to regulate
- 519 canonical Wnt/ $\beta$ -catenin signaling. These findings provide insights on the canonical/non-
- 520 canonical molecular events during WIHN. Furthermore, we also demonstrate that tissue
- 521 mechanics, or the stiffness of the wound bed, the ECM, and the stiffness of the cell also partake
- 522 during WIHN; the hair primordia formation is not exclusive to molecular signaling events. The
- 523 soft and easy-to-shed feature of the African spiny mouse skin not only serves as an escape
- 524 strategy from predators<sup>27</sup>, but also fosters the optimal mechanical cue for hair primordia
- formation during wound healing. Understanding the common and distinct features of laboratory
- and spiny mouse in response to wounding shed light on evo-devo advantages and provide
- 527 perspectives for future implications.

### 528 Methods

## 529 Animal model

530 All animal work was performed according to the approved animal protocol, guidelines and regulations for the care and use of laboratory animals of University of Southern California (USC). 531 Ethical approval was obtained for all experiments performed. All mice were housed in climate 532 controlled indoor facilities in a temperature range between 21 and 26 °C with a 12:12-h 533 534 controlled dark/light cycle. Humidity is maintained at 30% to 70%. C57BL/6J mouse purchased from Jackson Lab was used as the primary animal for this study. The K14-Cre-Twist1 mice were 535 bred by crossing the Twist1 conditional null (Twist1<sup>+/+</sup>)<sup>70</sup> and Tg(KRT14-cre)1Amc/J (Jackson 536 Lab) mice. The wild type Twist1<sup>+/+</sup> mice were used as the control in transgenic mouse study. 537 The African spiny mouse, Acomys cahirinus, is a kind gift from Dr. Malcolm Maden at the 538 539 University of Florida and Dr. Ashley W Seifert of University of Kentucky. A colony of captivebred Acomys cahirinus was established at USC, and all experiments were per formed with 540 541 protocols approved by the USC IACUC. We used both male and female 2-month-old spiny mice 542 and 4-week-old C57BI/6J mice for wound experiments unless otherwise specified.

543

### 544 Wound-induced hair neogenesis assay

545 Mice were anesthetized using Ketamine-Xylazine (80 mg/kg; 5 mg/kg) and analgesic

546 Buprenorphine SR (0.5 mg/kg) was given by intraperitoneal injection (IP) at the beginning of the

547 procedure. A 1x1 cm square full thickness wound was excised on the posterior dorsum of 4-

week-old mice (p28) using scissors, and let it heal by secondary intention. For the spiny mice,
 1.5x1.5 cm square full thickness wound was excised on 8 week-old mice. Additional DietGel

549 1.5x1.5 cm square full thickness wound was excised on 8 week-old mice. Additional DietGel
 550 Boost (ClearH2O) was placed on the bottom of the cage during the first week post-operation.

551 Mice of the same sex from the same litter were housed together and provided with half-dome

552 shelter.

553

554 Alkaline phosphatase (ALP) stain

To detect newly forming dermal papillae, alkaline phosphatase staining was performed as 555 previously reported<sup>22</sup>. Briefly, full thickness wounds were excised and epidermis separated from 556 557 the dermis using 20 mM EDTA. The dermis was fixed in acetone overnight at 4°C, and washed in PBS several times. The dermis was pre-incubated in ALP buffer (0.1 M Tris-HCI, 0.1 M NaCI. 558 5 mM MgCl<sub>2</sub> and 0.1% Tween-20) for 30 min, incubated with BCIP/NBT Color Development 559 Substrate (Promega, Madison, WI, USA) in ALP buffer at 37 °C until color development. The 560 561 reaction was stopped by washing with pH 8.0 Tris-EDTA and the tissue stored in PBS with sodium azide. 562

563

564 Atomic Force Microscopy

AFM (NanoWizard 4a/CellHesion, JPK, Berlin, Germany) was setup for contact mode
 indentation in PBS. The spring constants of all cantilevers were calibrated via thermal noise
 method with correction factor in liquid<sup>71,72</sup> prior to each measurement resulting in values of 0.03

N/m. To allow for proper modeling of the data, a glass bead (5  $\mu$ m in diameter) was attached at

the end of a tipless rectangular cantilever (Arrow-TL1, NanoWorld, Neuchatel, Switzerland)

using 2-component epoxy (Gorilla Glue Epoxy Clear, Gorilla Glue Company, Cincinnati, OH,

571 USA). A force series identified a maximum indentation force of 5 nN to show the most

572 consistent results on test samples. A constant rate of 1 μm/s was used for the entire approach

and retract sequence. Force-distance curves were collected and post-processed using the JPK

574 package software (Data Processing, 6.3.11). The force-curves were analyzed using the Hertz 575 model with a spherical indentation<sup>35,36</sup>

576 The force on the cantilever F(h) is given by:

$$F(h) = \frac{E_{sample}}{1 - v_{sample}^2} \frac{4\sqrt{R}}{3} h^{3/2}$$

577 where h is the depth of the indentation, E is the effective modulus of a system tip-sample, v is

the Poisson ratio for the sample, and R is the radius of the AFM tip. The unit of Young's

579 modulus is calculated as N/m<sup>2</sup>, and expressed as pascal (Pa) or kilopascal (kPa). Poisson ratio 580 was set at 0.5 since the spherical tip was incompressible relative to the sample. The

580 was set at 0.5 since the spherical tip was incompressible relative to the sample. The

temperature of the measurement was controlled at 32 °C to mimic the surface temperate of

582 mouse skin<sup>73</sup>.

583

To maintain the biomechanical force integrity of the dorsal wound, the entire mouse skin organ 584 was removed by creating an excision on the ventral side midline, extending from the anterior 585 neck region to the posterior genital region, and then dissecting away the skin organ from the 586 underlying fascia. Normal skin and wound stiffness were immediately measured after skin organ 587 removal to prevent artifacts from tissue decomposition. AFM measurements were positioned 588 589 across the wound starting from unwounded normal skin on one side and progressively traveling through to the opposite side; the near wound edge, the near wound bed, the wound center, the 590 opposite wound bed, the opposite wound edge, and the opposite normal skin. At least 5 591

indentation points were taken for each region of interest.

593

594 Second Harmonic Generation imaging

595 The animals were anaesthetized using Isofluorane. Body temperature was maintained with a 596 homeothermic blanket system (Harvard Apparatus, Holliston, MA, USA). The SHG images of 597 animals were acquired using the external detectors of an inverted Leica SP5 (Wetzlar, Germany) 598 multiphoton confocal fluorescence microscope powered by a Chameleon Ultra-II MP laser at 599 860 nm and a 40x Zeiss water-immersion objective (NA1.2). A Z-stack series of 3 µm per slice, 500 slices in total was recorded during a time course starting at the cornified layer of the 601 epidermis and ending at 150 µm depth for each time point.

602

## 603 Heatmap of spatial stiffness of the wound

The interpolation of tissue stiffness was performed by using 3-D meshgrid function of MATLAB (R2015b, Natick, MA, USA). After obtaining a Young's modulus (z) at a specific spatial location

(x,y) in the wound, a 3-dimensional matrix was defined. When the positions and stiffness of all

- the measured spots were identified, we could interpolate the stiffness of the positions in
- between to average the stiffness of the nearest parameters using 3-D meshgrid function, by
- defining (x,y) as meshgrid and (z) as griddata. In the end, the heatmap was generated by
- 610 defining the representative color of stiffness.
- 611
- 612 Wound area calculation and stiffness analysis
- The area of the wound is quantified by using the area measurement function under ImageJ
- according to its user guide ImageJ/Fiji 1.46 (NIH, Bethesda, MD). The photo of the wound was
- taken with a scale bar, hence the measured area in pixel unit can be converted to actual size.
- To obtain the area of the wound under 15 kPa, we specifically adjusted the (x,y,z) values
- according to each respective wound so the heatmap was also actual size. Using Photoshop, we
- use the wand tool and set the tolerance value to match that of 15 kPa on the scale bar. By using
- this parameter, the wand tool could select the area of the wound along the 15 kPa line. This
- selected area is then saved and quantified using ImageJ to obtain the final actual area.
- 621
- 622 Inhibitor Treatments

Blebbistatin (Cayman, MI, USA), Harmine (Cayman, MI, USA) and GM6001 (Cayman, MI, USA)

- 624 were dissolved in DMSO. 20 μl was applied once a day directly to the wound surface starting at
- post-wound day 10 (PWD10) and continuing until PWD16.
- 626
- 627 Histological preparations
- The wound tissues were fixed in 4% PFA and dehydrated in a graded alcohol series. The tissue
- 629 was cleared in Xylene and embedded in paraffin wax. 6 um sections were cut on a microtome.
- 630 H&E sections were performed according to accepted protocol. Whole-mount tissues were fixed
- in 4% PFA and then stored at 4°C in PBS with NaAzide.
- 632
- 633 Paraffin section and Whole-Mount Immunohistochemistry (IHC)

634 Fixed tissues were permeabilized with methanol and blocked with 3% H<sub>2</sub>O<sub>2</sub> for 30 min, and then serum blocked for 1h. The primary antibody was added and incubated over night at 4°C with 635 agitation. The tissue was washed with TBST (Tris Buffered Saline Tween 20) and the secondary 636 637 antibody was added for 1h at room temperature. The tissue was washed with TBST and if utilized, a tertiary antibody was added for 1 h at room temperature. The tissue was washed and 638 color was developed using the AEC kit (Vector Laboratories, CA, USA) or fluorescence was 639 visualized with a fluorescence microscope. The whole-mount samples were cleared in a series 640 of Glycerol-PBS until 100% Glycerol for imaging. The Twist1 (ab50887), Collagen I (ab34710) 641 and Collagen III (ab7778) antibodies are from Abcam (Cambridge, MA, USA), MMP9 (N2C1, 642 GTX100458) is from GeneTex (Irvine, CA, USA), Snai1 (13099-1-AP), P-cadherin (13773-1-AP), 643 E-cadherin (20874-1-AP) and Zeb2 (14026-1-AP) are from Proteintech (Rosemont, IL, USA). 644

The dilution ratio for section IHC was 1:50, 1:400 for wholemount immunostaining.

646

# 647 Harvesting wounds from laboratory mice for RNA extraction

648 The wound was harvested and a 3 mm diameter hole-punch biopsy was taken from the

649 geometric center of the wound, and the remaining wound tissue was considered the margin.

The epidermis and dermis were separated manually under a dissecting microscope. The

dissected dermis tissues were immediately placed in liquid nitrogen for 30 seconds. The frozen

- tissues were then disaggregated individually using a mortar and pestle, and then collected into a
   1.5 ml microtube. The dissected epidermis was also collected into a 1.5 ml microtube.
- 654
- 655 Harvesting wounds from spiny mice for RNA extraction

The spiny skin (shaved) or wound was harvested and placed epidermal side down in a 3 cm

657 culture dish filled with a thin layer of 0.25% Trypsin-EDTA (ThermoFisher, Waltham, MA, USA).

The level of trypsin should be just enough to cover the epidermis but not submerging the tissue.

The tissue is incubated at 4 °C for 6-12 h, rinsed in PBS and the dermis-epidermis were

660 separated manually under a dissecting microscope. The epidermis was collected into a 1.5 ml 661 microtube. The dissected dermis was placed in liquid nitrogen for 30 sec and disaggregated

661 microtube. The dissected dermis was placed in liquid nitrogen for 30 s 662 using a mortar and pestle, and collected into a 1.5 ml microtube.

- 663
- 664 RNA extraction and RNA-seq

The RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). 1 µg of total
RNA from each sample was used to construct an RNA-seq library using TruSeq RNA sample
preparation v2 kit (Illumina, CA, USA). Sequencing (75 cycles single-end or paired-end reads)
was performed by USC Molecular Genomics Core using a NextSeq 500 sequencer (Illumina,

- 669 CA, USA).
- 670
- 671 RNA-seq and microarray analysis

672 The mouse mm10 reference genome, and RefSeq genome annotation downloaded from the UCSC Genome Browser on 5 June, 2019 were used for RNA-Seg analysis <sup>74</sup>. The alignment, 673 quantification, normalization, and differentially expression analysis were performed by STAR 674 2.6.1d<sup>75</sup>, htseq-count 0.6.0<sup>76</sup>, TMM<sup>77</sup>, and edgeR 3.26.8<sup>78</sup>, respectively. P-value or False 675 discovery rate (FDR) < 0.05 was set as a threshold to identify differentially expressed genes 676 (DEG). The hierarchical clustering, Venn diagram, volcano plot, gene expression profile, and 677 678 scatter plot were carried out by Partek Genomics Suite 7.18.0723 (Partek Inc. MO, USA). The pathway enriched analysis based on Fisher's exact test, and upstream analysis were performed 679 by Ingenuity Pathway Analysis (Content Version: 60467501, Build: ing beryl, Date: 11-20-2020; 680 IPA, QIAGEN Inc. CA, USA). An HTMH form Perl CGI program was performed for the statistical 681 significance of the overlap between two groups of genes 682 683 (http://nemates.org/MA/progs/overlap\_stats.html). The gene set of DC signature gene and EMT core genes were build based on Supplementary Table 1 of <sup>79</sup> and up-regulated genes in 684

685 Supplementary Table 1 of <sup>80</sup>. Public microarray data under GEO database (GSE50418) were re-

analyzed using Partek Genomics Suite 7.18.0723 (Partek Inc. MO, USA).

687

# 688 Lentivirus production and transfection

The Twist1 overexpressing vector genome plasmid was cloned by inserting Twist1 promoter

690 into the lentiviral backbone: 5'LTR-cPPT-Ubq-eGFP-P2A-Twist1-WPRE-3'LTR. Twist1 promoter

691 was amplified from mouse genomic DNA, and the plasmid backbone was purchased from

- Addgene (Watertown, MA, USA). The empty backbone without Twist1 promoter insertion was
- 693 used as control.

694 293T cells (ATCC® CRL-3216<sup>™</sup>) at 50-60% confluency were transfected with 10 µg vector 695 genome plasmid, 10 µg of packaging construct ΔR8.2, and 2 µg envelope plasmid pCMV-VSVG 696 using the calcium phosphate method. 1 0mM sodium butyrate was added to fresh media 16 h 697 post-transfection and removed after 8 hours. Virus-containing media was collected at 36 h post-698 transfection, sterile filtered, and ultracentrifuged on a 20% sucrose cushion at 110,863 g and 699 4°C for 1.5 h before storing at -20°C for up to 30 days or -70°C indefinitely.

The virus was applied to the wound on PWD10 to infect the tissue. The efficiency of transfection

can be visualized by detecting the eGFP intensity under a fluorescent microscope, and later

- verified by frozen section IHC.
- 703
- 704 RT-qPCR

705 RNA extraction was done with Zymo Research Direct-zol RNA Kits. Reverse transcription was

done using Superscript III First Strand Synthesis kit. The RNA and cDNA concentrations were

707 measured with the NanoDrop 2000 spectrophotometer and normalized between samples.

Primers used for qPCR are listed in Supplementary Table 2. The Ct values were measured

- using the Agilent Mx3000P qPCR system. The relative quantification was done by pyQPCR
- 710 Version 0.9 software.
- 711

# 712 Statistics

The Kolmogorov–Smirnov tests were conducted to test normal distributed random samples.

Two independent sample T-tests (two-tailed) were used for comparing unpaired sample groups.

For some datasets not equally and normally distributed, Wilcoxon rank tests were conducted

using IGOR Pro or MATLAB to evaluate the statistically significant difference between two

samples. Chi-square test was conducted in MATLAB. The photographs are representative

samples of at least 4 replicates. Stiffness measurements were reported as the averaged of at

least 4 independent samples. Hair follicle counts are reported as the average from at least 6
 samples. Each bar on gPCR graph represents average and SE of 3 independent samples. All

samples. Each bar on qPCR graph represents average and SE of 3 independent samples. All
 data is presented as mean ± SD unless stated otherwise. Results from student t-tests (two-

tailed) with p < 0.05 was considered significant. \*, p < 0.05. \*\*, p < 0.01. \*\*\*, p < 0.005.

- 723
- 724

# 725 Data Availability

- The authors declare that all other data supporting the findings of this study are available within
- the article and its Supplementary Information files, or are available from the authors upon
- 728 request.
- Bulk RNA-seq data have been deposited in the NCBI Gene Expression Omnibus (GEO)
- 730 database under accession code: GSE159939
- 731 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159939 ].
- 732
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# 935 Author contributions

HIH, SPW, BVH, YC Liang, IMS, ST, SK, and TXJ performed the experiments. YCLai, AS, HX,

ADP analyzed the data. TEW generated the model. MH, MJT, JPP, DE, TXJ and CMC

- contributed study design and equipment support. HIH, YCLai, BVH, and CMC wrote themanuscript.
- 940

# 941 **Competing Interests**

- 942 The authors declare no competing interests.
- 943

#### 944 Figures legends

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946 Fig. 1. Tissue mechanics set up morphogenetic field for wound-induced hair follicle 947 neogenesis. a, A PWD28 C57BI/6 mouse. b, AP staining showing de novo hair follicle formation at the center of the wound bed at PWD28. c, Schematic diagram showing the location 948 of regenerated hair follicles (blue dots) in **b**. **d**, Stiffness heatmap of the overlaying PWD14 949 950 wound. Colorimetric unit: kPa. e, Cross-sectional view of the PWD14 wound and distribution of 951 wound stiffness. Yellow arrow heads indicate the formation of hair placodes. Heatmap and number indicate the spatial stiffness of the whole wound bed. Unit: kPa. Green arrow indicates 952 953 the range of the morphogenetic field. f, A Bleb-treated mouse on PWD28. g, AP stain showing the number of hair follicles increased significantly upon Bleb treatment. h, Schematic diagram of 954 hair follicles (blue dots) in g. i, Stiffness heatmap of PWD14 Bleb-treated overlaying wound. j, 955 Dot plot showing changes in HF upon Bleb treatment. n = 8 biologically independent animals. 956 Data are presented as mean values  $\pm$  SEM. p = 0.003, unpaired two-sided *t*-test. **k**. The area of 957 958 the wound bed under 15 kPa positively correlates with the number of hair follicles. SHG of I PWD14 and **m** PWD21 wounds. The color squares indicate the location of corresponding 959 960 enlarged photos from **n**, PWD14 center, **o**, PWD14 margin, **p**, PWD21 center and **q**, PWD21 961 margin of the wound image. r, Dot plot showing the number of fibers per square unit. PWD14 962 C/M: p = 0.0323, PWD14/PWD21 C: p = 0.0495, PWD21 C/M: p < 0.0001. s, Fiber thickness in respective wound time and location. PWD14 C/M: p = 0.0454, PWD14/PWD21 C: p = 0.0020, 963 964 PWD21 C/M: p < 0.0461. **r-s**, Data are presented as mean values  $\pm$  SD. n = 8 regions examined over 4 biologically independent animals. One-way ANOVA, Tukey test. t, Summary graph of 965 wound stiffness on PWD14 and PWD21 with respect to wound location and Bleb treatment. n = 966 10 regions examined over 5 biologically independent animals per location per condition. Data 967 are presented as mean values ± SD. \*, p = 0.0478; \*\*, p = 0.0061; \*\*\*, p < 0.0001. One-way 968 ANOVA. Tukey test, u. Illustration of tissue mechanics partakes in setting up the morphogenetic 969 field for WIHN. Red line: wound margin. Green: soft, morphogenetic field. Blue: hair placodes. 970 Ctl: control; Bleb: Blebbistatin; HF: hair follicle; sq: square; Lt: left; Rt: right; Morph: 971 972 morphogenetic. The images in a-i represent 8 out of 8 experiments performed. The images in I-973 q represent 4 out of 4 experiments performed.

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976 Fig. 2. African spiny mouse exhibits an optimal range of tissue stiffness for placode 977 formation. a, A spiny mouse on PWD28. b, K15 wholemount immunostaining of a PWD28. spiny mouse wound. Red line: border of the wound bed. c. Schematic diagram of hair follicles 978 (blue dot) in **b**. **d**, The stiffness heatmap of the overlaying PWD14 spiny mouse wound. 979 Colorimetric unit: kPa. e, H&E histology and stiffness heatmap of PWD14 and f PWD21 wounds. 980 The color and number indicate the stiffness of the wound at respective location. Unit: kPa. (e1-981 e3) enlarged images of regions 1, 2 and 3 from e. g, H&E of the PWD28 wound. Yellow arrows 982 indicate the formation of hair placode. h, SHG of PWD14 and PWD21 spiny mouse wounds. 983 Red line: wound border. i, IHC of collagen I and collagen III in spiny mouse at different post-984 wound time. i, Blebbistatin treatment significantly reduced the resultant number of hair 985 986 neogenesis. n = 5 biologically independent animals. Data are presented as mean values ± SEM. p < 0.0001, unpaired two-sided *t*-test. **k**, Graph indicating the respective wound stiffness of the 987 specific wound location and time in the laboratory and spiny mice. n = 10 regions examined 988 989 over 5 biologically independent animals per location per condition. Data are presented as mean values ± SD. ###, p < 0.0001 when compared between Mus PWD14 and Acomys PWD14; \*, p = 990 0.0482 when compared between Mus PWD14 and Mus/Bleb PWD14 center, p = 0.0218 when 991 992 compared between Mus PWD14 and Mus/Bleb PWD14 at the right of wound center, p = 0.0441 between Acomys PWD14 and Acomys PWD21 at the left of wound center. p = 0.0381 when 993 994 compared between Acomys PWD14 and Acomys/Bleb PWD14 wound margin right, p = 0.0421 at center-right, \*\*, p = 0.0089; \*\*\*, p < 0.0001. One-way ANOVA, Tukey test. I, Illustration of 995 996 morphogenetic field in PWD14 and PWD21 spiny mouse wound bed. Red: wound border. 997 Green: morphogenetic field. ###: P<0.005. Ctl: control; Bleb: Blebbistatin; Lt: left; Rt: right; Morph: morphogenetic. The images in a-g, i represent 5 out of 5 experiments performed. The 998 999 images in h represent 4 out of 4 experiments performed.

### 1001 Fig. 3. Transcriptome analysis identifies Twist1 as an important transcription factor

1002 during WIHN in laboratory mouse. a, Gene expression heatmap of PWD14 epidermis wound

- 1003 center vs wound margin. **b**, The significantly enriched pathways of DEG. **c**, Volcano plot
- showing gene expression fold changes of representative DEG in MMP, Twist1, Wnt/β-catenin
   related pathways, proliferation of epithelial cells, cell movement of epithelial cells. Upregulation
- 1006 indicates high expression in wound center. FC: fold change. C/M: wound center versus margin.
- 1007 **d**, Twist1 ranked 2<sup>nd</sup> by two-sided Fisher's Exact Test p-value in the top 10 differentially
- 1008 expressed upstream regulator. **e**, IPA identifies Twist1 as the top upstream regulator of the
- 1009 downstream DEG (p =  $1.1 \times 10^{-21}$ ). f, Wholemount immunostaining of Twist1 at respective
- location from the epidermal side of the PWD14 wound as illustrated. Red line: wound border.
   Green line: morphogenetic zone. Blue dots: hair placode. g, IHC of Twist1 and Snai1 in PWD14
- 1012 wounds containing the hair placode. Dotted line demarcates the border of dermal epidermal
- junction. Blue: Hoechst. Scale bar: 100  $\mu$ m. (a-d), n = 2 biologically independent experiments.
- 1014 The images in **f-g** represent 4 out of 4 biologically independent experiments performed.

#### 1016 Fig. 4. Twist1 is also expressed and can modulate the outcome of WIHN in spiny mouse.

1017 **a**, Gene expression heatmap of spiny mouse epidermis at different post-wound days. **b**,

- 1018 Proliferation of epithelial cells and cell movement associated genes in the spiny mouse wound
- 1019 epidermis. **c**, Twist1 related transcription factors in the spiny mouse wound dermis. **d**, ECM
- remodeling in the spiny mouse wound dermis. **e**, IHC of Twist1, Zeb2, MMP9, E-cad and P-cad
- in PWD14 the regenerating hair placodes of the spiny mice. White arrows point to the
- downregulation of E-cad at the tip of hair placode. **f**, The effects of Twist1 inhibitor Harmine and
- pan-MMP inhibitor GM6001 treatment on hair neogenesis in spiny mouse. Red: wound border;
   yellow arrow: hair follicles. Observed on PWD35. g, Dot graph of the number of regenerated
- hair follicles (HF) observed in spiny mouse wounds under different treatments. n = 6 biologically
- independent animals. Data are presented as mean values  $\pm$  SEM. \*, p = 0.0265; \*\*, p = 0.0027;
- 1027 unpaired two-sided *t*-test. Ctl: control. The images in **f** represent 6 out of 6 experiments
- 1028 performed. The images in **e** represent 4 out of 4 experiments performed.

1030 Fig. 5. Epidermal Twist1 regulates both epidermal and dermal cell behavior and tissue stiffness toward hair primordia formation in WIHN. a, A K14-Cre-Twist1 mouse with a 1031 1032 PWD28 wound. **b**, AP staining of a K14-Cre-Twist1 PWD28 wound. **c**, Schematic diagram of **b**. d, Stiffness heatmap of PWD14 K14-Cre-Twist1 wound overlaying on the wound photo. e-g, AP 1033 staining of control (Ctl), and Harmine and GM6001 treated wounds on PWD28. h-i, Schematic 1034 1035 diagrams of e-g, respectively. j, Photo of PWD28 wound transfected with Twist1-overexpressing lentivirus. **k**, bright field and **I**, fluorescence image of GFP-tagged-Twist1 overexpressing (oe) 1036 virus in the PWD28 wound, colocalizing with hair follicles. **m**, Schematic diagram of **k** and **l**. **n**, 1037 1038 Dot plot showing the resultant hair follicle number in wild type, K14-Cre-Twist1, Harmine-treated, 1039 GM6001-treated and Twist1-overexpressing (oe) virus treated PWD28 wounds. n = 7 biologically independent animals. Data are presented as mean values ± SEM. All comparisons 1040 1041 made to WT. Twist1-oe: p = 0.0074, K14-Cre-Twist1: p = 0.0008, Harmine: p < 0.0001, GM6001: 0.0044. Unpaired two-sided *t*-test. **o**, Changes in wound stiffness upon different perturbations. 1042 K14-Cre-Twist1, GM6001 and Harmine treatments all significantly increased the stiffness of the 1043 wound center region, marked by asterisks. Green bar: 5-15 kPa morphogenetic range. n = 5 1044 1045 biologically independent animals. Data are presented as mean values ± SEM. All comparisons 1046 made to WT of respective location on wound. Center: K14-Cre-Twist1, p = 0.0006; GM6001, p = 0.0006; Harmine, p = 0.0018. Center-left: K14-Cre-Twist1, p = 0.01115; GM6001, p = 0.0137; 1047 1048 Harmine, p = 0.0214. Center-right: K14-Cre-Twist1, p = 0.0083; GM6001, p = 0.006; Harmine, p 1049 = 0.0159. Unpaired two-sided *t*-test. **p**, gPCR analysis: ECM remodeling related-gene 1050 expression fold change (FC) of wild type epidermis wound center vs K14-Cre-Twist1 wound center. n = 3 biologically independent samples. Data are presented as mean values ± SEM. All 1051 1052 comparisons made to WT. *Mmp2*, p = 0.0483; *Mmp9*, p = 0.0014; *Mmp13*, p = 0.0005; *Itga1*, p = 0.0071. Unpaired two-sided *t*-test. **q**, PWD14 wild type vs K14-Cre-Twist1 dermis wound 1053 center RNA-seg analysis identifies significantly enriched pathway affected by K14-Cre-Twist1 1054 knockout. r. Dermal condensation (DC) signature genes downregulated in PWD14 K14-Cre-1055 Twist1 dermis wound center. Red dotted line: wound boarder. Yellow arrow: hair follicle. 1056 1057 Harmine: Twist1 inhibitor. GM6001: pan-MMP inhibitor. Ctl: control. The images in a-m represent 6 out of 7 experiments performed. 1058

Fig. 6. Multiscale tissue mechanics set up morphogenetic field for WIHN. a. AFM stiffness 1060 mapping demonstrates the stiffness of the hair placode. This is based on experimental data. 1061 Top left: an AFM cantilever scanning a PWD14 wound. Blue box demarcates the 100 x 100 µm 1062 scanning area. Below: stiffness heatmap of a placode. Colorimetric unit: kPa. Right panel: Dot 1063 plot showing the stiffness of placode with respect to wound margin and center. Representative 1064 1065 image of 3 out of 3 experiments. n = 9 regions examined over 3 independent biological animals. Data are presented as mean values ± SD. p < 0.0001, unpaired two-sided *t*-test. a', Schematic 1066 diagram of placode stiffness and its respective cross-sectional view. b, Hypothetical model 1067 1068 showing feedback loop in a Turing system with an underlying measure of stiffness. Simulations illustrating stationary distributions of *u* (activator, blue background blocks) and *E* (stiffness, pick 1069 1070 background blocks) with increasing s (source coefficient, measures the strength of the E 1071 source). The underlying domain is a square of side length 100 and the color shows the value of S at each grid point, S(x, y). The 'size' of the soft region is controlled by  $\sigma$ . From left to right and 1072 then the row below, the values of s are 2, 4, 6, 8 and 10 and  $\sigma$  = 20. In all cases random initial 1073 conditions from a uniform distribution of [0.5,1.5] are used. c, Schematic drawing showing the 1074 1075 opposite topology of morphogenetic competent and non-competent region in the wound bed. d, 1076 Conceptual summary of the way we perceive the relationship between tissue stiffness and 1077 morphogenetic field (Top). Summary based on data from Fig. 1 and 2. Middle: It highlights the 1078 different geographic distribution of the morphogenetic field (green) within a wound bed (red 1079 frame), and also the periodic appearance of hair primordia (orange) within the morphogenetic 1080 field (green). When the wound stiffness is too low (blue), no placode can form. Bottom: Stiffness 1081 of different wound beds predicate distribution of morphogenetic field and placode formation.

# Table 1. The significantly enriched pathways in PWD14 epidermis wound center vs margin RNA-seq analysis

Enriched Pathway	p-value	Differentially expressed genes
Wnt/β-catenin signaling (X30)	1.49 X 10 <sup>-2</sup>	appl2, bmpr2, cdh5, csnk1d, dkk3, dvl1, ep300, fzd2, gsk3b, hdac1, kremen2, lrp1, lrp6, ppard, ppp2r1a, ppp2r2c, ppp2r3a, ppp2r5b, ptpa, sfrp1, sox15, sox18, src, tgfb1, tgfb3, tle3, wnt16, wnt10a, wnt10b, wnt5a
Regulation of EMT Pathway (X28)	1.02 X 10 <sup>-2</sup>	dvl1, fzd2, hgf, lox, map2k2, mmp2, mmp9, notch4, pard6b, pdgfrb, ralb, smad3, smurf1, snai1, tgfb1, tgfb3, twist1, twist2, wnt10a, wnt10b, wnt5a, wnt9b
MMP genes (X1)	4.53 X 10 <sup>-7</sup>	adam12, hspg2, lrp1, mmp2, mmp3, mmp9, mmp10, mmp11, mmp13, mmp14, mmp17, mmp19, mmp23b, sdc1, thbs2, timp1, timp3
Organization of ECM (X84)	1.32 X 10 <sup>-23</sup>	adam12, adamts4, agrn, apbb2, aplp1, bgn, bmp1, c6orf15, col16a1, col18a1, col1a1, col1a2, col27a1, col3a1, col4a1, col4a2, col5a1, col5a2, col5a3, col6a1, col6a2, col6a3, col6a4, col7a1, col8a1, ctsk, dcn, ddr1, egflam, elf3, emilin1, fbln5, fbn1, fbn2, fn1, furin, ibsp, icam2, itga1, itga11, itga3, itga5, itga9, itgb3, itgb6, jam2, kdr, lama4, lox, lox11, mfap2, mmp1, mmp10, mmp11, mmp13, mmp14, mmp19, mmp2, mmp3, mmp9, nid1, nid2, olfml2a, olfml2b, pdgfra, pecam1, postn, prdx4, ptx3, pxdn, serpine1, sh3pxd2b, sparc, spp1, timp1, tnc, tnf, vcam1, vcan, vtn, vwf
Proliferation of epithelial cells (X186)	3.47 X 10 <sup>-17</sup>	ahr, alms1, areg, atm, bad, bcl11b, birc2, bnc1, brca1, calm1, casp3, casp8, ccnd3, cd9, cdc25b, cdc73, cdkn1a, cdkn1b, cebpa, cers2, col8a1, creb3l3, cryab, csf2rb, ctsv, cul3, cxcr2, dab2ip, eif4e, eng, ep300, epgn, epha2, ercc1, ereg, esrra, ezh2, fbln5, fgfr2, fn1, frs2, fst, gata3, glul, grn, gsk3b, has2, hbegf, heyl, hgf, hoxa5, hyal1, ifngr1, ift52, ift74, ift80, igf1, il18, il22ra2, il24, il4r, il6r, inhba, inhbb, itga1, itga3, itgb3, junb, kcnk2, kdr, klf10, klf5, klk3, klk6, klk8, krt16, krt17, lgals7/lgals7b, lgr4, lmnb1, lrp6, maged1, map2k1, map2k6, map2k7, mapk7, mapk8, mapk9, mapkapk2, marveld3, mfge8, mki67, mmp14, mmp9, mt2, nab1, nab2, nfib, nfkbia, nme2, npm1, nr3c1, nrg1, odc1, p2rx7, pkp3, postn, ppard, prlr, ptafr, pten, ptgs2, pthlh, ptpn1, ptprk, rack1, rbl2, rela, relb, rgn, rida, rps6kb1, s1pr2, sema4d, serpinf1, sfn, sfrp1, sh2b1, slc20a1, slc7a5, smad3, smad7, snai2, socs1, socs3, sparc, spint2, spp1, stat5a, stmn1, tfrc, tgfb1, tgfb3, tgm1, timeless, timp1, tnfaip6, tnfrsf11a, tnfrsf12a, tnfrsf1a, tslp, twist1, twist2, uhrf1, vegfa, wnt10b, wnt16, wnt5a, yod1, zbtb16

1085

1086 Comparison using two-sided Fisher's Exact Test.

1088	Table 2. The 20 significantly upregulated genes related to hair placode formation at
1089	epidermis wound center comparing to wound margin on PWD14.

Gene	Description	Location	Type(s)
cdh11	cadherin 11	Plasma Membrane	other
meg3	maternally expressed 3	Other	other
twist1	twist family bHLH transcription factor 1	Nucleus	transcription regulator
col1a1	collagen, type I, alpha 1	Extracellular Space	other
col3a1	collagen, type III, alpha 1	Extracellular Space	other
col6a2	collagen, type VI, alpha 2	Extracellular Space	other
col6a3	collagen, type VI, alpha 3	Extracellular Space	other
efemp2	EGF containing fibulin-like extracellular matrix protein 2	Extracellular Space	other
fbn1	fibrillin 1	Extracellular Space	other
fstl1	follistatin like 1	Extracellular Space	other
lgals1	lectin, galactoside-binding, soluble, 1	Extracellular Space	other
timp2	TIMP metallopeptidase inhibitor 2	Extracellular Space	other
cpxm1	carboxypeptidase X (M14 family), member 1	Extracellular Space	peptidase
dpysl3	dihydropyrimidinase like 3	Cytoplasm	enzyme
ftl1	ferritin, light polypeptide	Cytoplasm	enzyme
pde5a	phosphodiesterase 5A	Cytoplasm	enzyme
ppic	peptidylprolyl isomerase C	Cytoplasm	enzyme
myh10	myosin, heavy chain 10, non-muscle	Cytoplasm	other
srpx2	sushi-repeat containing protein, X-linked 2	Cytoplasm	other
igf2bp2	insulin like growth factor 2 mRNA binding protein 2	Cytoplasm	translation regulator

1092Table 3. The 114 significantly upregulated transcription factors at epidermis wound1093center comparing to wound margin on PWD14.

асар3	cers2	fam129b	hr	mier2	relb	sreb†1	wtip
aes	churc1	fem1a	id3	mllt1	rfx1	srebf2	zbtb42
arid3a	cic	fiz1	ier2	mnt	rfxank	srf	zdhhc13
arid5a	cited4	fosl1	ifi204	mxd1	rnf114	ssbp4	zfp219
asb1	creb3l3	fosl2	irf1	nab2	rnf25	tbx15	zfp369
asb6	csrnp1	foxc1	irf5	nfatc4	rnf4	tbx3	zfp444
atf4	ctbp2	foxp4	irf7	nfkbia	sbno2	tcf23	zfp593
atf6b	dnmt3l	glis2	irx1	noct	scand1	tcfl5	zkscan6
atxn7l3	e2f7	glmp	jarid2	notch4	siah2	thap4	zxdc
barx2	eaf1	gpank1	junb	pax9	smad3	trim16	
bhlhe40	ehmt2	hdac1	klf10	phf1	smad7	tsc22d1	
bnc1	elf3	helz2	lmo1	pitx1	snai1	tsc22d4	
btg2	elk1	heyl	lztr1	prrx1	sox15	twist1	
carhsp1	elk3	hic2	maff	rbpms	sox18	twist2	
cebpa	esrra	hopx	maged1	rela	sqstm1	ube2v1	

1095 Based on p-value < 0.05













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Regulation of EMT Pathway (X28)	1.02 X 10 <sup>-2</sup>	dvl1, fzd2, hgf, lox, map2k2, mmp2, mmp9, notch4, pard6b, pdgfrb, ralb, smad3, smurf1, snai1, tgfb1, tgfb3, twist1, twist2, wnt10a, wnt10b, wnt5a, wnt9b
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col6a3	collagen, type VI, alpha 3	Extracellular Space	other	
efemp2	EGF containing fibulin-like extracellular matrix protein 2	Extracellular Space	other	
fbn1	fibrillin 1	Extracellular Space	other	
fstl1	follistatin like 1	Extracellular Space	other	
lgals1	lectin, galactoside-binding, soluble, 1	Extracellular Space	other	
timp2	TIMP metallopeptidase inhibitor 2	Extracellular Space	other	
cpxm1	carboxypeptidase X (M14 family), member 1	Extracellular Space	peptidase	
dpysl3	dihydropyrimidinase like 3	Cytoplasm	enzyme	
ftl1	ferritin, light polypeptide	Cytoplasm	enzyme	
pde5a	phosphodiesterase 5A	Cytoplasm	enzyme	
ppic	peptidylprolyl isomerase C	Cytoplasm	enzyme	
myh10	myosin, heavy chain 10, non-muscle	Cytoplasm	other	
srpx2	sushi-repeat containing protein, X-linked 2	Cytoplasm	other	
igf2bp2	insulin like growth factor 2 mRNA binding protein 2	Cytoplasm	translation regulator	

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arid3a	cic	fiz1	ier2	mnt	rfxank	srf	zdhhc13
arid5a	cited4	fosl1	ifi204	mxd1	rnf114	ssbp4	zfp219
asb1	creb3l3	fosl2	irf 1	nab2	rnf25	tbx15	zfp369
asb6	csrnp1	foxc1	irf5	nfatc4	rnf4	tbx3	zfp444
atf4	ctbp2	foxp4	irf7	nfkbia	sbno2	tcf23	zfp593
atf6b	dnmt3l	glis2	irx1	noct	scand1	tcfl5	zkscan6
atxn7l3	e2f7	glmp	jarid2	notch4	siah2	thap4	zxdc
barx2	eaf1	gpank1	junb	pax9	smad3	trim16	
bhlhe40	ehmt2	hdac1	klf10	phf1	smad7	tsc22d1	
bnc1	elf3	helz2	lmo1	pitx1	snai1	tsc22d4	
btg2	elk1	heyl	lztr1	prrx1	sox15	twist1	
carhsp1	elk3	hic2	maff	rbpms	sox18	twist2	
cebpa	esrra	hopx	maged1	rela	sqstm1	ube2v1	

Table 3. The 114 significantly upregulated transcription factors at epidermis woundcenter comparing to wound margin on PWD14.

Based on p-value < 0.05